

Identification and Characterization of a Virulence-
Associated Gene in *Shigella dysenteriae*

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Shigella species, including *Shigella dysenteriae*, are causative agents of bacillary dysentery, a disease characterized by severe diarrhea and blood in the stool. Although a number of genes required for virulence have been characterized in *S. dysenteriae*, others remain to be identified. These additional virulence-associated genes can be identified by screening mutants of *S. dysenteriae* in a plaque assay. The plaque assay is used to infer virulence by measuring the ability of the bacteria to invade, grow within, and spread between eukaryotic cells, resulting in the formation of plaques, small holes in a monolayer of eukaryotic cells. Mutants that have lost the ability to form normal plaques are necessarily avirulent.

A previously described non-directed mutant, SDU380, lost the ability to form plaques. Further characterization revealed that this strain had sustained a 33 kilobase deletion. None of the genes in the deleted region are known virulence genes. The purpose of this study was to identify and characterize a novel virulence gene in this deleted region that resulted in the inability of SDU380 to form plaques. Different mutant strains missing only some of the genes deleted in SDU380 were constructed and tested for virulence in a plaque assay. This analysis revealed that the loss of the gene *yciB* is responsible for the failure of SDU380 to form plaques. The role of the protein YciB in *S. dysenteriae* virulence is unknown, though the *yciB* gene is a known virulence-associated gene in *Shigella flexneri*, a species with a similar lifestyle to that of *S. dysenteriae*. In *S. flexneri*, YciB plays a role in septation, an essential step in cell division. Further analysis showed that loss of YciB in *S. dysenteriae* also results in an intracellular growth defect. However, this defect does not seem to be due to loss of ability to undergo septation and no growth defect is observed *in vitro*.

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I. INTRODUCTION

A. General Background

Shigellosis, infection with *Shigella* species, is the major type of bacillary dysentery, a disease caused by bacteria and characterized by severe diarrhea and blood in the stool (7, 12, 15). It causes approximately 1.1 million deaths worldwide each year (7). Shigellosis affects primarily children under the age of five and, with about 164 million cases a year, is endemic in developing countries (2, 15). Due to increased antibiotics resistance of *Shigella* species, the World Health Organization has prioritized vaccine development against *Shigella dysenteriae* and *Shigella flexneri*, two of four *Shigella* species (7, 15).

Humans are the only natural host of *Shigellae* (7, 15, 19). The bacteria can only be transmitted via the fecal oral route, either through direct contact or through contaminated food or water (15, 16). The prevalence of shigellosis in developing countries is therefore thought to be attributable to poor hygiene and poor sanitation (7).

Shigellae are gram negative bacteria, a classification based on staining characteristics, and are closely related to *Escherichia coli* (15). The genus *Shigella* includes four species, *Shigella flexneri*, *Shigella boydii*, *Shigella sonnei* and *Shigella dysenteriae* (7). *Shigella dysenteriae* is responsible for shigellosis epidemics in developing countries (15). It causes a particularly severe form of shigellosis through the production of Shiga toxin, a substance that causes a disease called hemolytic uremic syndrome which may lead to acute kidney failure (15, 30). Shiga toxin may also cause paralysis (15). *S. flexneri* is the predominant endemic species of *Shigella* in developing countries, and *S. sonnei* is responsible for most *Shigella* infections in industrialized countries (7, 15). The manifestation of the disease also depends on the immune status of the host and the presence of other risk factors such as infection with HIV, further

accounting for the pervasiveness of shigellosis in developing countries and among children (3, 15).

B. Pathogenesis

With an infectious dose of only 10 to 100 bacteria, *Shigella* species are extremely virulent (7, 15). The site of pathogenesis is the human colon, where *Shigella* infection results in inflammation, a non-specific immune response, and destruction of the colon epithelium, a single layer of cells facing the lumen (interior) of the colon and acting as a barrier to bacteria (12, 15). The destruction of the epithelium leads to the characteristic symptoms of shigellosis, including watery diarrhea, abdominal pain, and blood in the stool (7, 19).

Shigellae are usually ingested via a food or water source and, because they are highly acid resistant, can pass undamaged through the stomach and small intestine. Once in the colon, *Shigellae* go through three stages of pathogenesis: invasion of human colonic epithelial cells, replication within these cells, and spread to infect neighboring cells (Fig. 1) (15). This results in the death of host epithelial cells (15). Genes necessary for these three stages are usually found in one of three pathogenicity islands (sections of the chromosome) or on a virulence plasmid (7).

1. Invasion of epithelium

Shigellae invade human epithelial cells from the basolateral side (Fig. 1), the side away from the lumen (7, 19). To access the basolateral side of the intestinal epithelium, the bacteria cross the epithelial layer through one of three mechanisms.

- The colonic epithelial layer contains M cells, specialized cells that endocytose (take up) samples of the contents of the intestinal lumen and transport these samples to the other side of the epithelial layer to a pocket filled with white blood cells, cells of the immune system (7). This arrangement forms an integral part of the immune system, as it allows the body to prepare for pathogens before the infection can occur.

Shigellae, like some other pathogens including *Salmonella* and *Yersinia*, are able to

take advantage of this system by using M cells to cross the epithelial layer (7, 19).

Once phagocytosed (taken up) by macrophages (a type of white blood cell) in the pocket on the basolateral side of the intestinal epithelium, *Shigella* turns on genes (primarily *ipaD*) that cause lysis of the phagocytic vacuole and apoptosis (cell death) of the macrophage (7). This results in the bacteria being released into the sub-mucosa, the space on the basolateral side of the epithelial layer (7). This is the main form of *Shigella* invasion.

- Apoptosis of macrophages also induces inflammation, recruiting more cells of the immune system to the site of infection (7, 19). Some of these cells, called PMN cells (polymorphonuclear leukocytes), squeeze between the epithelial cells, creating gaps in the epithelial layer (7). Once again, *Shigellae* take advantage of the host defenses by crossing the epithelial layer through the gaps created by the PMN cells (19).
- *Shigellae* can also cross the epithelial layer by manipulating proteins in tight junctions, structures anchoring adjacent epithelial cells to each other (7). Thus, the bacteria create gaps and cross the epithelial layer independently of the immune response system as well.

Once on the basolateral side of the epithelial layer, *Shigellae* stimulate epithelial cells to engulf them (7, 19). This is achieved by inducing a rearrangement of the cytoskeletal network of filaments that gives structural support to the epithelial cell. This results in the formation of membrane extensions surrounding the bacteria, internalizing the pathogen (7, 19). To accomplish this, *Shigellae* bind receptors ($\alpha 5\beta 1$ and CD44) on the epithelial cell (19). These receptors are associated with the actin cytoskeleton, suggesting that the initial binding of the bacteria to the epithelial cell begins the process of cytoskeleton rearrangement (19). The

receptors are bound by proteins (IpaB-C-D) on the surface of the bacterium (19). Upon binding the bacterium inserts a two-protein complex (IpaB and IpaC) into the epithelial cell membrane, forming a pore through which components of a type III secretion system (encoded by the *mxi-spa* locus) transport two proteins (IpaA and IpaC) into the epithelial cell (7, 19). These two proteins coordinate the cytoskeleton rearrangement that results in the engulfment of the bacteria (7, 19). Once internalized, *Shigellae* use IpaB to lyse the membranous vacuole that is formed as a result of the engulfing, releasing the bacteria into the host epithelial cell cytoplasm (Fig. 1) (19).

2. Intracellular Replication

Once inside the host epithelial cell, *Shigellae* multiply rapidly, doubling in number every 40 minutes (7). Replication includes growth of the bacterium, replication of the genetic material contained within the bacterium, equal distribution of the replicated genetic material to different parts of the cell (binary fission), followed by division (septation) of the bacterium resulting in two equivalent daughter cells.

3. Intracellular and Intercellular Spread

Shigellae move within the host epithelial cell and into neighboring cells by manipulating the host cytoskeletal system (Fig. 1). *Shigellae* express and localize a protein (IcsA) to one pole of the bacterium, where it interacts with a host protein (N-WASP), stimulating polymerization (growth) of actin, a filament of the cytoskeleton system (7). Actin polymerization on one pole of the bacterium propels the bacteria through the interior of the host epithelial cell until it encounters the plasma membrane (structure that surrounds each epithelial cell) and forms a protrusion into the epithelial cell adjacent to the already infected host cell (7). This protrusion containing *Shigellae* is endocytosed by the neighboring cell, resulting in a double membraned

vesicle containing the bacteria inside this epithelial cell (7). Once again *Shigella* lyses the membranes of the vacuole it is contained within (IpaB and IpaC mediated) (7). Free in the new host epithelial cell, the bacteria continue to replicate and spread.

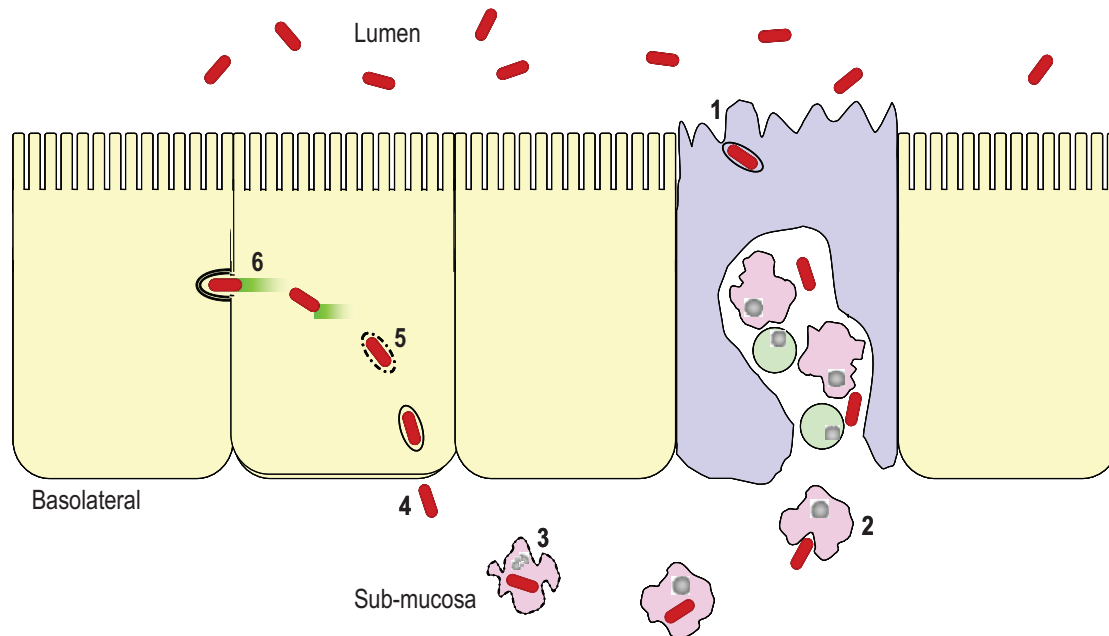


Figure 1: *Shigella* pathogenesis (4)

1. M-cell dependent invasion of host epithelial layer. 2. Uptake of *Shigellae* by macrophages on the basolateral side of the epithelium. 3. Release of *Shigellae* into the sub-mucosa following *Shigellae* induced macrophage apoptosis. 4. *Shigellae* stimulated uptake by epithelial cell. 5. *Shigellae* induced evasion of membranous vacuole releasing the bacteria into the host cytoplasm. 6. Intracellular and intercellular spread as a result of actin polymerization

4. Epithelial cell death

Cell death or necrosis of host epithelial cells was initially thought to be a result of overpacking of *Shigellae* in host cells due to continuous replication of the bacteria (7). It is now, however, thought that the death of host epithelial cells is caused by the inflammatory response of the host attacking and killing infected epithelial cells (7). The destruction of the epithelial layer in turn leads to the characteristic symptoms of shigellosis (15).

C. Plaque Assay

Since the only natural host of *Shigellae* are humans, *Shigellae* virulence cannot be studied using animal models (7, 15, 19). Virulence can, however, be inferred using the plaque assay (Fig. 2 left side) (19). In this assay, a monolayer of human epithelial cells is infected with *Shigellae* and then incubated for 4 days. The plaque assay tests the ability of the bacteria to invade, grow intracellularly, and spread from cell-to-cell, three essential steps of *Shigella* virulence (7, 15). If the bacteria are successful in all three of these activities, epithelial cell death results in the formation of plaques, small clearings within the monolayer. A strain that can form wild-type (normal) plaques is not necessarily virulent, since other factors, such as sensitivity to pH of the stomach, may prevent the bacteria from reaching the colon. Mutants that have lost the ability to form plaques (Fig. 2 right side) are, however, necessarily avirulent as the steps required for plaque formation are essential to the pathogenesis of *Shigella* species (7, 15).

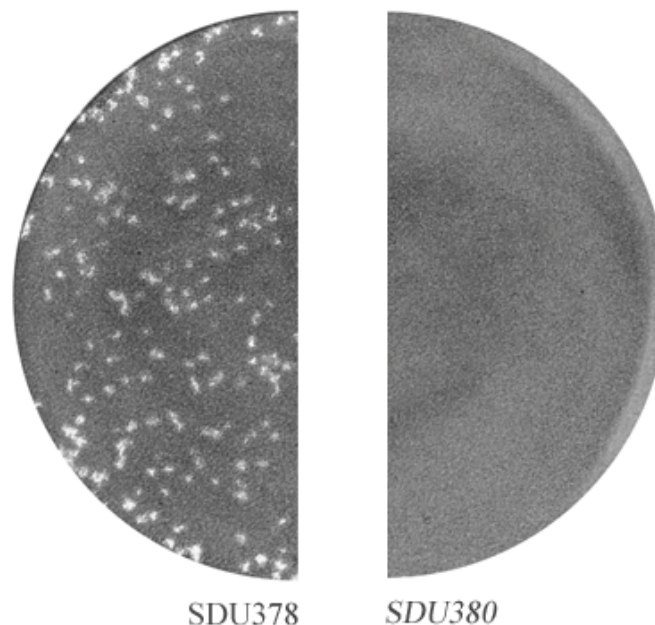


Figure 2: SDU380, a spontaneous mutant of clinical isolate SDU378, has lost the ability to form plaques.

D. Avirulent strain SDU380

Though many of the genes involved in *Shigella* pathogenesis are known, others remain to be identified. To find these genes, *Shigella* mutants may be screened for loss of the ability to form wild-type plaques in the plaque assay and then analyzed to identify virulence-associated genes responsible for the mutant phenotype.

SDU380 is a *S. dysenteriae* mutant that has lost the ability to form plaques (Fig. 2) (23). SDU380 was isolated by selecting for pirazmonam-resistance among spontaneous mutants of clinical isolate SDU378 (23). Pirazmonam is an antibiotic (anti-bacterial agent) that is taken up by bacteria using a particular high-affinity iron transport system (TonB). Once taken up, the pirazmonam kills the bacteria (23). Bacteria that survived pirazmonam treatment must therefore have a mutation that prevented pirazmonam uptake. SDU380 is thus a spontaneous mutant of SDU378 that is deficient in TonB function.

Further analysis of the mutant revealed that SDU380 had sustained a large deletion of approximately 33 kilobases, about 1% of the *S. dysenteriae* genome. The deleted region spans from *oppA* to *yciL* and includes *tonB*, the gene encoding TonB, the oligopeptide permease (*opp*) operon, the tryptophan biosynthesis (*trp*) operon, the *yciCBA* operon and a number of uncharacterized genes (Fig. 3). One or more of the genes contained in this deleted region is likely responsible for the failure of SDU380 to form plaques and is thus a virulence-associated gene.

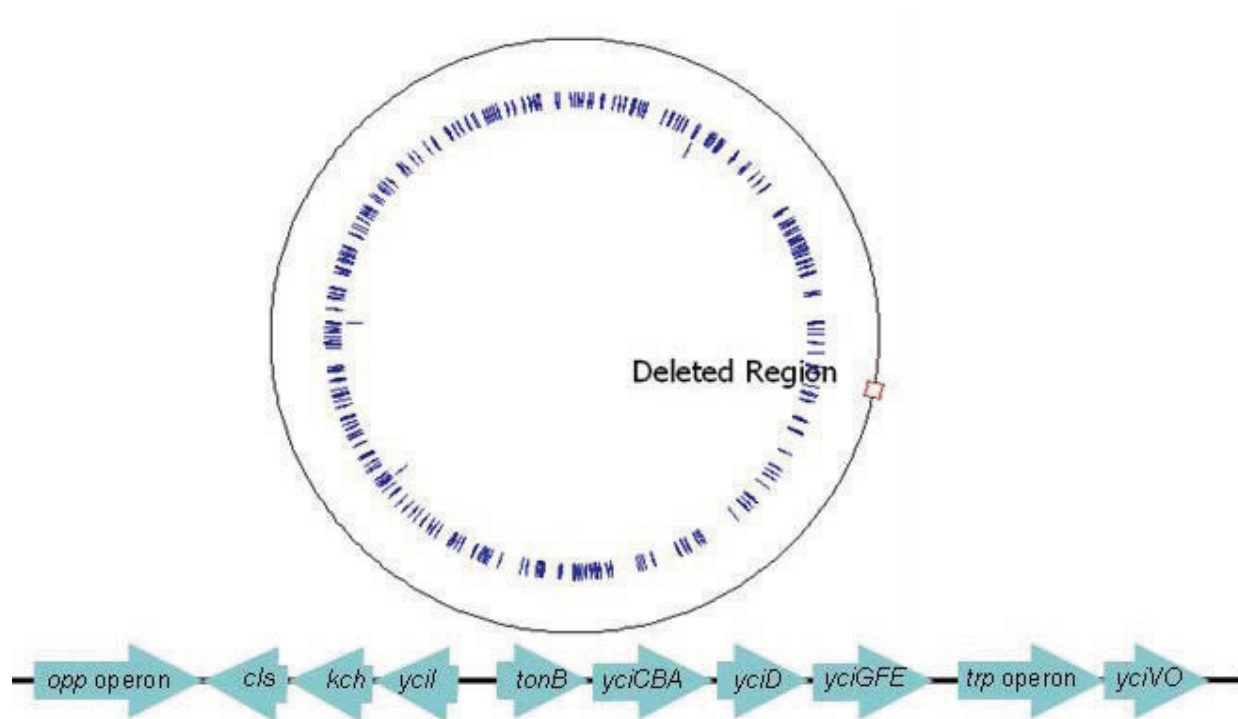


Figure 3: Deleted region in SDU380

1. Genes in Deleted Region in order of chromosomal location

a. Oligopeptide permease (*opp*) operon

This operon (series of genes that are co-regulated and code for proteins that usually function together) makes up a substantial part of the deletion of SDU380. It encodes five proteins (OppA, OppB, OppC, OppD, OppF) that form a transport system for small peptides (10). As such this operon is important in cell-cell signaling, transport of peptides as carbon and nitrogen sources, cell wall component recycling, and many other important bacterial functions (9, 20).

Although no research studies have shown a virulence-associated role of the oligopeptide permease system in *Shigella*, several studies have revealed that the *opp* operon is involved in the pathogenesis of other bacteria. In many bacterial species the oligopeptide permease complex is involved in quorum sensing, a process to monitor bacterial population density and that may be

involved in virulence initiation (9). In *Vibrio fluvialis*, another human pathogen, the oligopeptide permease operon appears to be involved in biofilm production. Biofilms are formed by aggregates of bacteria that function together to protect one another and that are involved in many diseases (10). Another study revealed a role for the products of the *opp* operon in the pathogenesis of group A streptococci, a Gram positive human pathogen (9, 10, 31).

b. Cardiolipin synthase (*cls*)

Cardiolipin synthase is an enzyme that catalyzes the last step in the synthesis of cardiolipin, a lipid found in cell membranes (29). Though *Escherichia coli* lacking this enzyme were found to be less viable when the bacteria have reached maximal density and have an increased doubling time, no evidence exists to suggest that *cls* is a virulence-associated gene in *E. coli* or another bacterial species (29).

c. Potassium channel (*kch*)

This channel allows for bacterial uptake of potassium within the host epithelial cell (18). The *kch* gene shares substantial homology with potassium channel genes in eukaryotic cells (18). Although no studies have shown a role in virulence for this potassium channel in *Shigella* species, a study has identified a similar protein, SapG, in *Salmonella typhimurium* that when deleted results in reduced virulence in a mouse model of infection (18).

d. Putative gene *yciI*

This gene is poorly characterized. It may encode a protein involved in cell morphology since this is the putative role of a *yciI* homologue in *Haemophilus influenzae* (32). No studies have revealed a role of *yciI* in pathogenesis.

e. TonB

Shigellae need to acquire many essential nutrients such as iron to survive. Inside the host epithelial cell, *Shigellae* are dependent on the host cell for these essential nutrients (23, 28).

There are several iron transport systems that *Shigella* uses to transport iron from the host epithelial cell cytoplasm into the bacterium (23, 28). TonB-dependent systems are a major type of iron transport system used both outside and inside host cells (23). TonB is involved in the transport of iron complexes across the outer bacterial membrane, one of two membranes surrounding all Gram negative bacteria. TonB is associated with outer membrane receptors and transduces the energy required to transport iron complexes across the outer membrane (23, 28).

TonB was shown to be involved in the virulence of several *Salmonella* species, *Vibrio* species, *Pseudomonas* species, *Haemophilus* species, and *Escherichia coli* (28). However, a *S. dysenteriae* mutant lacking *tonB* was found to be able to form plaques in a plaque assay, indicating that *tonB* is not responsible for the no-plaque phenotype of SDU380 (4).

f. Putative operon *yciCBA*

While *yciC* and *yciA* are uncharacterized, *yciB*, also called *ispA*, is a known virulence-associated gene in *S. flexneri* (6, 12). YciB is a putative membrane protein (6, 12). *S. flexneri* lacking *yciB* were found to have lost the ability to form plaques in a plaque assay, indicating that *yciB* is essential for *S. flexneri* virulence (6, 12). Further analysis of the *S. flexneri yciB* mutant showed that this strain is avirulent due to a septation (cell division) deficiency as well as an actin polymerization deficiency (6, 12). The inability to properly divide results in the formation of long filaments of partially attached bacteria that presumably hinders intercellular spread. The inability to polymerize actin causes the bacteria to be unable to generate the propelling force

necessary to spread to neighboring cells (6, 12). There have been no studies conducted to investigate the possible role of *yciB* (*ispA*) in *S. dysenteriae*.

g. Putative gene *yciD*

This gene encodes a putative outer membrane protein. In *Salmonella*, *yciD* was shown to encode a cell envelope protein (24). There are no studies that have shown a role of *yciD* in bacterial pathogenesis.

h. Putative gene *yciE*

This gene codes for a stress protein (11). Stress proteins are a set of proteins that are expressed by organisms under particular stress condition for increased protection of the organism and are known to play a role in virulence (22)

i. Tryptophan Biosynthesis (*trp*) operon

This operon also makes up a substantial part of the deletion in SDU380. As the name suggests, the tryptophan biosynthesis operon encodes proteins that are involved in the synthesis of the amino acid tryptophan from precursors in the absence of tryptophan in the bacterial environment.

Though there is no evidence suggesting that the tryptophan biosynthesis operon is involved in *Shigella* pathogenesis, a study has shown that tryptophan is involved in the virulence of pathogenic *E. coli* against both *Caenorhabditis elegans* (microscopic worms) and humans (1). Tryptophan, either acquired from the environment or through biosynthesis, was shown to be involved in the regulation of Shiga toxin production and of pathogenicity gene expression in pathogenic *E. coli* (1).

j. Putative operon *yciVO*

These two genes are not well characterized. Crystal structure analysis suggests that *yciO* codes for an RNA binding protein (8). YciV is a putative enzyme of unknown function. Neither gene is known to play a role in pathogenesis.

k. Putative gene *yciL*

This gene is also known as *rluB* and encodes a pseudouridine synthase, an enzyme that catalyzes the conversion of uridine to pseudouridine in RNA molecules, in particular rRNA and tRNA (5). No studies have revealed a role for *yciL* (*rluB*) in pathogenesis.

E. Purpose of this study

Given the prevalence of shigellosis and *Shigella* in developing countries and the recent increase of antibiotic resistance among these bacteria, *Shigella* species have been the subject of many studies. This is particularly true for *S. dysenteriae*, the leading cause of bacillary dysentery epidemics and responsible for a particularly severe form of shigellosis (15). *Shigella* pathogenesis is one of the main foci of these studies, and one of the main objectives is to identify and characterize the genes and proteins involved in *Shigella* pathogenesis. The purpose of this study is to identify and characterize a novel virulence-associated gene in *S. dysenteriae* through the analysis of the spontaneous avirulent mutant SDU380.

II. MATERIALS AND METHODS

A. Bacterial Strains, Plasmids, and Oligonucleotides

Bacterial strains used in this study are listed in Table 1. Table 2 contains a list of plasmids used in this study. Oligonucleotides (primers) used are listed in Table 3. Primers were designed using Clone Manager Professional Software Suite version 8 (Scientific and Educational Software, Cary, NC) and synthesized by Integrated DNA Technologies, Coralville, IA. Primers were diluted to 100 μ M using distilled water and stored at -20°C.

Table 1: Bacterial strains used in this study

Strain name	Description	Reference or source
DH5 α	<i>E. coli</i> strain	(27)
SY327 λ pir	Contains the prophage λ pir	(13)
MM294	Conjugation helper strain	R. Meyer, Univ. Texas, Austin
SDU378	<i>S. dysenteriae</i> Type I clinical isolate	(23)
SDU380	spontaneous pirazmonam resistant SDU380 mutant	(23)
0-4576S1	Derivative of <i>S. dysenteriae</i> clinical isolate	(14)
0-4576S1-G	spontaneous streptomycin resistant 0-4576S1 mutant	(4)
0-4576S1-GW	spontaneous avirulent 0-4576S1-G mutant	E.R. Murphy, Univ. Texas, Austin
NDS115	0-4576S1-G <i>tonB::cat</i>	(4)
KHS100	0-4576S1-G <i>trpEDC::kan</i>	This work
KHS101	0-4576S1-G <i>oppABC::chl</i>	This work
KHS103	0-4576S1-G <i>yciB::chl</i>	This work

Table 2: Plasmids used in this study

Plasmid	Description	Reference or source
pGEM-T Easy	Cloning vector	Promega
pHM5	Allelic exchange vector	(25)
pQE2	Expression vector, IPTG inducible	Qiagen
pKH1	Splice overlap PCR of <i>S. dysenteriae</i> O-4576S1-GW <i>trpEDC</i> cloned into pGEM-T Easy	This work
pKH2	PCR of <i>S. dysenteriae</i> O-4576S1-GW <i>oppABC</i> cloned into pGEM-T Easy	This work
pKH3	Kanamycin resistance gene from pUC4K cloned into the <i>SmaI</i> site of pKH1	This work
pKH5	<i>XbaI EcoRV</i> fragment (<i>trpEDC::aph</i>) of pKH3 cloned into pHM5	This work
pKH7	Chloramphenicol resistance gene from pMTL24Cam cloned into the <i>SmaI</i> site of pKH2	This work
pKH8	<i>XbaI SphI</i> fragment (<i>oppABC::chl</i>) of pKH7 cloned into pHM5	This work
pKH12	Splice overlap PCR of <i>S. dysenteriae</i> O-4576S1-G <i>yciCBA</i> cloned into pGEM-T Easy	This work
pKH13	Chloramphenicol resistance gene from pMTL24Cam cloned into the <i>SmaI</i> site of pKH12	This work
pKH14	<i>SaI EcoRV</i> fragment (<i>yciCBA::chl</i>) of pKH13 cloned into pHM5	This work
pRZ526	Plasmid carrying <i>E. coli yciCBA</i> and <i>tonB</i>	(21)

pND55	Plasmid carrying <i>E. coli</i> <i>yciB</i>	N.M.L. Davies, Univ. Texas, Austin
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Table 3: Primers used in this study

Primer Name	Sequence
trp.1	GCCAGATTGATATCTACCCAATTGCCGG
trp.2	CACCGCCAGTCCCGGGTAAGGAGTTCTGGC
trp.3	GAACTCCTTACCCGGGACTGGCGGTGACGG
trp.4	CGCTTTCTTGCACTCTAGAATAAACGCCG
opp.3	TACCCGCATGCGTCACACTGG
opp.4	TAGCGATGTCTAGACCACCACC
yciB.3	CGCTACGCAGATATCAACTCGATCG
yciB.4	GGATTTATCTTCCCGGGCTTCATTTTACGATTCCG
yciB.5	GAAGCCCGGGAAGATAAATCCTAACC
yciB.6	CTGCTGATCTAGAACCGC

B. Media and Growth Conditions

E. coli strains were cultured in Luria-Bertani broth (LB broth) (10g tryptone, 5g yeast extract and 10g NaCl per liter) at 37°C or grown on Luria-Bertani agar (LB agar) at 37°C. *S. dysenteriae* strains were cultured in LB broth at 30°C or at 37°C or grown on tryptic soy broth agar (TSB agar) plus 0.01% Congo red dye at 37°C.

Antibiotics were added to LB broth as follows: 250 µg/ml of carbenicillin, 50 µg/ml of kanamycin, 35 µg/ml of chloramphenicol, and 200 µg/ml of streptomycin. Strains containing a *tonB* mutation were supplemented with 40 µM FeSO₄. Isopropyl-β-d-thiogalactosidase (IPTG) was used to induce expression of genes cloned behind an inducible plasmid promoter.

C. General Molecular Techniques

1. Recombinant DNA Methods

The QIAprep Spin Miniprep kit (Qiagen, Santa Clarita, CA) was used to isolate plasmid DNA as indicated by the manufacturer. The QIAquick gel extraction kit (Qiagen) was used to isolate DNA fragments from agarose gels as described in the manufacturer's instructions.

Enzymes used for endonuclease restriction digests and DNA ligations were purchased from New England Biolabs (Beverly, MA) and Promega (Madison, WI). The reactions were performed as described by Sambrook and Russel (27). *Hind*III or *Bst*EII digested λ DNA was used as size markers to estimate DNA fragments separated by gel electrophoresis.

2. Polymerase Chain Reaction (PCR)

PCR reactions were carried out in an Applied Biosystems GeneAmp thermocycler or an MJ Research PTC-200 thermocycler using *Taq* polymerase (Qiagen) as indicated the manufacturer. A 100 μ L PCR reaction contained the following: template DNA, 5 units of *Taq* polymerase, 10 μ M of each primer, 250 μ M of each dNTP, and 1x *Taq* reaction buffer. Small scale 20 μ L PCR reactions at the same relative concentrations were carried out for screening purposes. The following PCR program was used: 1) 5 minutes at 94°C; 2) 30 seconds at 94°C; 3) 30 seconds at 50°C; 4) 1 minute per 1000 bp of expected PCR fragment size at 72°C; 6) repeat steps 2 through 4 29 times; 7) 7 minutes at 72°C.

3. DNA Sequencing

Mutations in bacterial strains were verified by DNA sequencing using the automated dye termination procedure. Sequences were analyzed on an ABI 377A DNA sequencer by the Core Facility at the Institute for Cellular and Molecular Biology at the University of Texas at Austin.

4. Transformation of Bacterial Strains

a. Electroporation of *Shigella*

This technique, as described by Sambrook and Russel (26) was used to introduce plasmids into electrocompetent bacterial strains. Bacteria were made electrocompetent as follows: Strains were grown till mid-log at 30°C in two milliliter LB broth containing appropriate antibiotics and chilled on ice for one hour. Bacteria were harvested by 6000x centrifugation at 4°C for 10 minutes and washed with 25 ml of ice cold water. Centrifugation was repeated and bacteria were washed with 10 ml of ice cold water. This was repeated one more time with 5 ml of ice cold water. After a final centrifugation bacteria were resuspended in 250 µL of ice cold water plus 10% glycerol. One hundred µL of electrocompetent cells were used per electroporation.

b. Conjugation

S. dysenteriae mutants were constructed by allelic exchange. Plasmids were introduced into *S. dysenteriae* by conjugation. This was achieved by centrifuging one ml each of overnight cultures of donor strain containing the plasmid with the mutated allele, recipient strain, and helper strain and resuspending each pellet in 100 µL LB broth. Twenty µL of each suspension was mixed and dotted onto an LB agar plate. The plate was incubated at 37°C for 6-8 hours. The bacteria were collected with a swab and resuspended in 1 ml LB broth. Bacteria were plated onto agar plates containing appropriate antibiotics to select for successful transconjugants.

D. Tissue Culture

Henle cells (intestinal 407 cells) purchased from the American Type Culture Collection, Manassas, Va. were used for all cultured cell experiments. Cells were grown in Henle medium (Eagle's minimum essential medium, 2 mM glutamine, and 10% fetal bovine serum) in a 5% CO₂ atmosphere at 37°C.

1. Invasion Assays

To determine the stage of pathogenesis for which a putative virulence gene is essential, invasion assays were performed. General instructions as described by Hong et al. (6) were followed with the following adjustment: the monolayer of Henle cells was incubated for 15 minutes following infection before medium containing extracellular bacteria was removed and replaced by medium containing 20 µg/ml gentamicin to kill any remaining extracellular bacteria. Monolayers were stained with Wright-Giemsa stain (Baxter Scientific Products, McGaw Park, Ill.) and observed under the microscope.

2. Plaque Assays

Plaque assays were performed to infer virulence of *S. dysenteriae* mutants. General instructions are described by Oaks et al. (17) with the modifications by Hong et al. (6) and the following adjustments: monolayers of Henle cells were grown to confluence in 35 mm diameter plates (approximately 2×10^6 cells per plate) and infected with 2×10^4 bacteria. Medium was removed after 60 minutes and replaced with medium containing 0.45% (weight/volume) glucose and 20 µg of gentamicin per mL. Plaque assays were incubated for 48 to 96 hours. Medium was removed and monolayers were stained with Wright-Giemsa stain.

E. *In vitro* Competition Assays

1. Colony Size Assay

This assay was used to compare mutant and wild type *in vitro* growth on LB agar. Bacteria were grown overnight and diluted. Approximately 100 bacteria were plated on LB agar and incubated overnight at 37°C. Bacteria carrying a complemented plasmid were grown in the presence of 0, 1, 10, or 100 µM IPTG. Using a microscope, the size of 10 colonies per plate was measured and the mean was calculated and compared.

2. End Point Analysis

This technique was used to compare mutant and wild type *in vitro* growth in LB broth. Three overnight cultures of each bacterial strain were diluted approximately 1:100 so that each culture contained an equal number of bacteria. Bacteria containing a complementing plasmid were grown in the presence of 0, 1, 10, or 100 µM IPTG. Cultures were grown at 37°C for approximately 8 hours and absorbance was measured at 600 nm.

3. Growth Assay

This technique was used to further investigate *in vitro* growth rates in LB broth. Bacteria were grown as described in the section labeled End Point Analysis with the following modifications: the optical density (A_{650}) of each culture was measured at the 2, 3, 4, 5, 6, 7, and 24 hours. Bacteria containing a complementing plasmid were grown in the presence of 1 µM or 100 µM IPTG.

F. Construction of Mutant Strains

1. KHS100

The *S. dysenteriae* *trp* mutant was constructed as follows: Using primers *trp*.1 and *trp*.2 and primers *trp*.3 and *trp*.4, two fragments containing parts of *trpE* and *trpD* and parts of *trpD* and *trpC* were amplified from O4576S1-GW. The two fragments were combined using overlap-extension PCR with primers *trp*.1 and *trp*.2, creating a *SmaI* site within *trpD*. The resulting PCR fragment was cloned into pGEM-T Easy and designated pKH1. pKH1 contains a unique *XbaI* site upstream of the cloned fragment and a unique *EcoRV* site downstream of the fragment. A *HincII* fragment of pUC5K carrying the *aph* gene (Kan resistance) was inserted into the *SmaI* site internal to *trpD*, yielding pKH3. After double digestion of pKH3 with *XbaI* and *EcoRV*, the cloned fragment was inserted into *XbaI* *EcoRV* digested pHM5, yielding pKH5. pKH5 was transferred into O4576S1-G via conjugation. Recombination and allelic replacement was confirmed by PCR analysis of sucrose-resistant, carbenicillin-sensitive, kanamycin-resistant isolated colonies. The resulting *trpEDC::kan* strain was designated KHS100. DNA sequence analysis verified successful mutant construction.

2. KHS101

The *S. dysenteriae* *opp* mutant was constructed as follows: Primers *opp*.3 and *opp*.4 were used to amplify part of *oppA*, *oppB*, and part of *oppC* from O4576S1-GW. The resulting PCR fragment was cloned into pGEM-T Easy and designated pKH2. The plasmid contains a *SphI* site downstream of the cloned fragment and a unique *XbaI* site upstream the fragment in addition to a *SmaI* site internal to *oppB*. A *SmaI* fragment of pMTL24Cam carrying the *cat* gene (Chl resistance) was inserted into the *SmaI* site in *oppB*, yielding pKH7. The cloned fragment containing the *cat* gene was removed from pKH7 using *SphI* and *XbaI* and inserted into *SphI*

XbaI digested pHM5, yielding pKH8. pKH8 was transferred into O4576S1-G via conjugation and recombination and allelic replacement was confirmed by PCR analysis of sucrose-resistant, carbenicillin-sensitive, chloramphenicol -resistant isolated colonies. The resulting *oppABC::chl* strain was designated KHS101 and DNA sequence analysis verified successful mutant construction.

3. KHS103

The *S. dysenteriae yciB* mutant was constructed as follows: Using primers yciB.3 and yciB.4 a DNA fragment containing parts of *yciC* and *yciB* was amplified from O4576S1-G. Another fragment containing part of *yciB*, *yciA*, and part of *tonB* was amplified from O4576S1-G using primers yciB.5 and yciB.6. The two fragments were combined using overlap-extension PCR with primers yciB.3 and yciB.6, creating a deletion and a *SmaI* site within *yciB*. This PCR fragment was cloned into pGEM-T Easy and designated pKH12. The plasmid contains a unique *SalI* site upstream of the cloned fragment and an *EcoRV* site downstream of the fragment. A *SmaI* fragment of pMTL24Cam carrying the *cat* gene (Chl resistance) was inserted into the *SmaI* site in *yciB*, yielding pKH13. The *SalI* and *EcoRV* fragment of pKH13 (the cloned fragment) was inserted into *SalI* *EcoRV* digested pHM5, yielding pKH14. pKH14 was transferred into O4576S1-G via conjugation and recombination and allelic replacement was confirmed by PCR analysis of sucrose-resistant, carbenicillin-sensitive, chloramphenicol-resistant isolated colonies. The resulting *yciB::chl* strain was designated KHS103. DNA sequence analysis verified successful mutant construction.

III. RESULTS

A. Identification of novel virulence gene in *S. dysenteriae*

1. Background

To identify which of the genes deleted in SDU380 accounts for the loss of ability to form plaques, mutants lacking only one gene or one operon were constructed and tested for the ability to form plaques in a plaque assay. The virulence gene could not be identified by simply complementing SDU380 with plasmids carrying only one of the genes in the deleted region, because SDU380 carries resistance genes to nearly all common antibiotics.

2. *oppABC* is not required for plaque formation in *S. dysenteriae*

A *S. dysenteriae oppABC* mutant, KHS100, was constructed and tested in a plaque assay. The mutant was able to form plaques in similar number and of similar size as the wild type parental strain, indicating that the lack of the oligopeptide permease complex in SDU380 was not responsible for the no-plaque phenotype of the strain (Fig. 4).

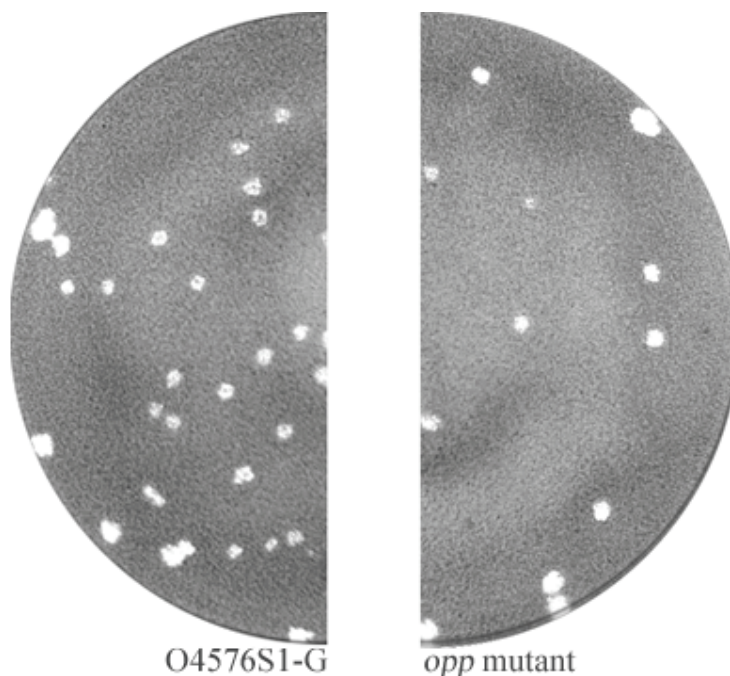


Figure 4: The *S. dysenteriae* oligopeptide permease mutation is not responsible for the no-plaque phenotype of SDU380.

A *S. dysenteriae* mutant lacking *oppABC* and the *S. dysenteriae* wild-type parental strain were tested in a plaque assay. There was no observable difference in plaque number and size, indicating that gene(s) other than in the oligopeptide permease operon are responsible for the loss of ability of SDU380 to form plaques.

3. ***trpEDC* is not required for plaque formation in *S. dysenteriae***

A *S. dysenteriae* *trpEDC* mutant, KHS101, was constructed and tested in a plaque assay.

The mutant strain was able to form wild-type plaques, indicating that genes other than the ones encoding the tryptophan biosynthesis system are responsible for the no-plaque phenotype of SDU380 (Fig. 5).

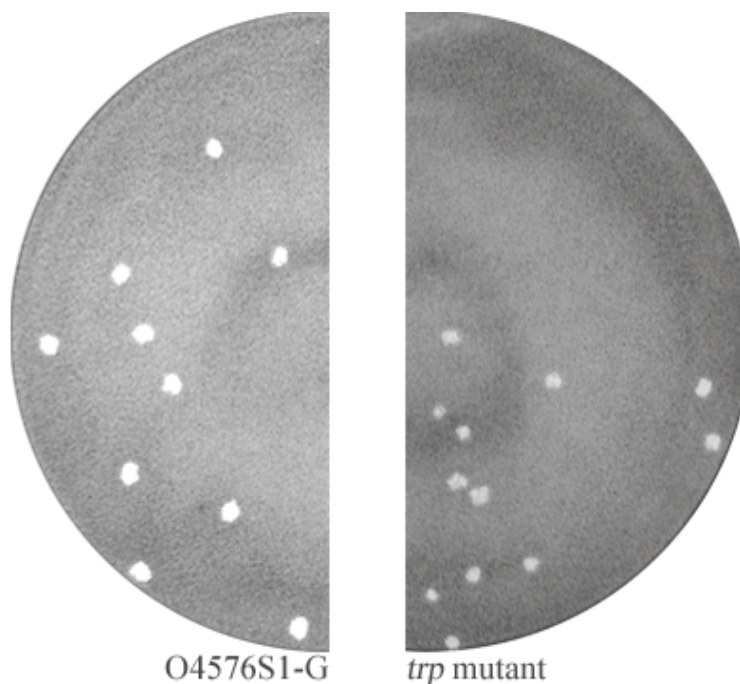


Figure 5: The *S. dysenteriae* tryptophan biosynthesis deletion is not responsible for the no-plaque phenotype of SDU380.

A *S. dysenteriae* mutant lacking *trpEDC* and the *S. dysenteriae* wild-type parental strain were tested in a plaque assay. Both strains formed plaques of approximately same size and number, indicating that gene(s) other than in the tryptophan biosynthesis operon account for the loss of ability to form plaques of SDU380.

4. *yciB* is a virulence gene in *S. dysenteriae*

A *S. dysenteriae yciB* mutant, KHS103, was constructed and tested in a plaque assay.

The mutant was not able to form plaques, indicating that *yciB* is a virulence gene (Fig. 6).

Complementing KHS103 with a plasmid carrying *yciB*, pND55, restored the ability to form plaques when *yciB* was expressed at low levels (0 μ M IPTG, 1 μ M IPTG and 10 μ M IPTG).

Overexpression of *yciB* with 100 μ M IPTG resulted in loss of ability to form plaques, indicating that *yciB* expression is tightly controlled in *S. dysenteriae* (Fig. 7).

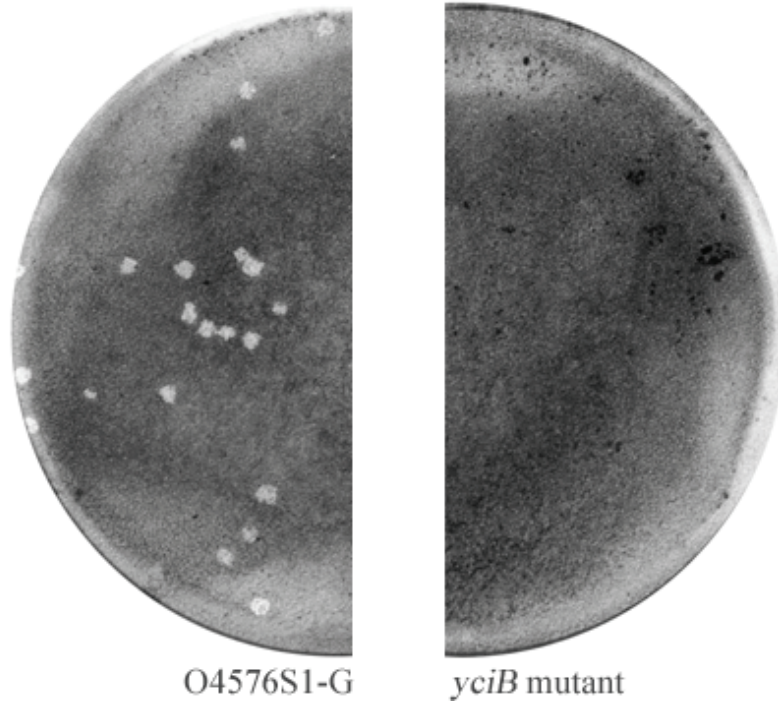
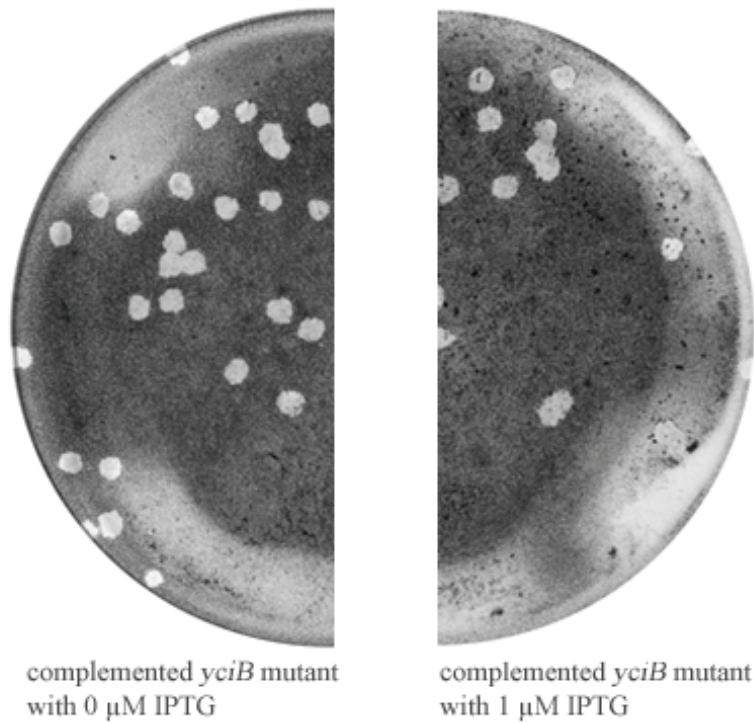


Figure 6: *yciB* is a *S. dysenteriae* virulence gene.

A *S. dysenteriae* mutant lacking *yciB* and the *S. dysenteriae* wild-type parental strain were tested for virulence in a plaque assay. The mutant did not form plaques, indicating that it is avirulent and that *yciB* is a virulence gene.



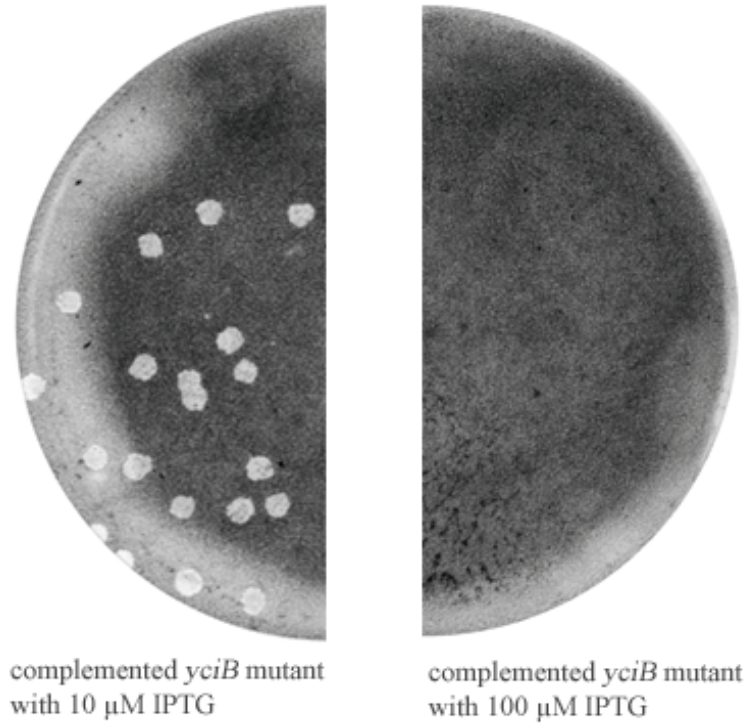


Figure 7: The *S. dysenteriae yciB* gene is tightly regulated.

Complementing the *S. dysenteriae yciB* mutant with *yciB* restored the ability to form plaques when the plasmid was not induced or only slightly induced (0 μ M IPTG, 1 μ M IPTG, or 10 μ M IPTG). Overexpression of *yciB* in the presence of 100 μ M IPTG did not complement plaque formation.

B. Characterization of virulence gene *yciB*

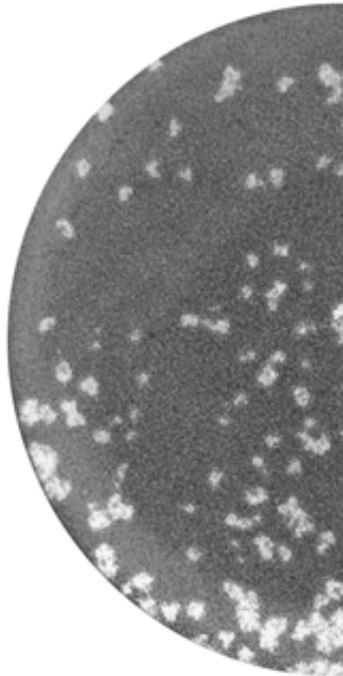
1. Background

As briefly mentioned in the introduction, *yciB* is a known virulence gene in *S. flexneri*. A *S. flexneri* mutant lacking *yciB* is therefore unable to form plaques and further characterization of the mutant revealed that this is likely due to both an intracellular growth defect and an intracellular spread defect (12). The mutant forms long filaments that appear to be unable to completely septate. The mutant is also unable to spread intracellularly, a step in pathogenesis that depends on the bacteria's ability to polymerize actin.

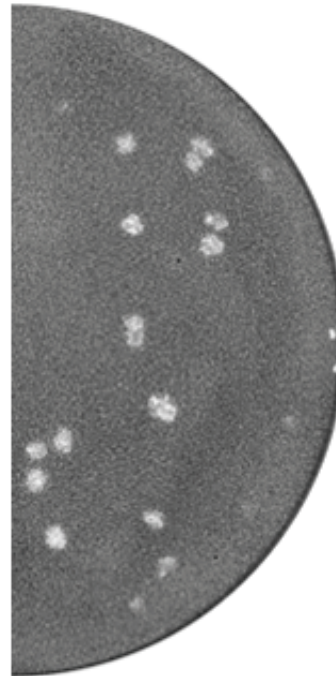
The loss of ability of the *S. dysenteriae* mutant to form plaques in a plaque assay indicates that *yciB* is a virulence gene. Complementation studies of SDU380 with *yciB* were done to determine whether *yciB* is the only virulence gene in the deleted region and an invasion assay was performed to study during which stage of *S. dysenteriae* pathogenesis YciB is essential.

2. YciB complements plaque formation of SDU380

Complementing SDU380 with a plasmid carrying *yciB* restored plaque formation if *yciB* was expressed in low levels (0 μ M IPTG or 1 μ M IPTG) (Fig. 8). This indicates that only the *yciB* gene was responsible for the no-plaque phenotype of SDU380. Expression of *yciB* at higher levels (10 μ M IPTG or 100 μ M IPTG) did not complement the plaque phenotype, further indicating *yciB* is highly regulated in *S. dysenteriae* (Fig. 8).



SDU380 complemented with
yciB with 0 μ M IPTG



SDU380 complemented with
yciB with 1 μ M IPTG



SDU380 complemented with
yciB with 10 μ M IPTG



SDU380 complemented with
yciB with 100 μ M IPTG

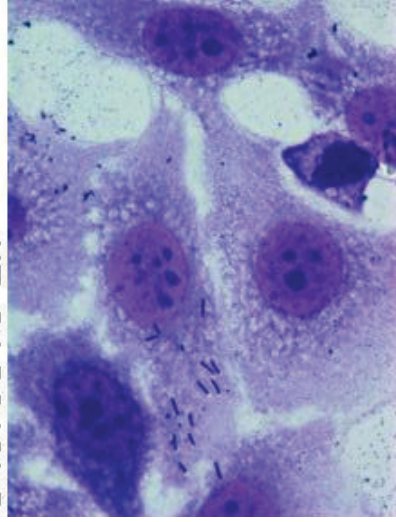
Figure 8: The *S. dysenteriae yciB* gene is tightly regulated.

Complementing SDU380 with *yciB* restored the ability to form plaques when *yciB* was not induced or only slightly induced (0 μ M IPTG or 1 μ M IPTG). Overexpression of *yciB* (10 μ M IPTG or 100 μ M IPTG) did not complement plaque formation.

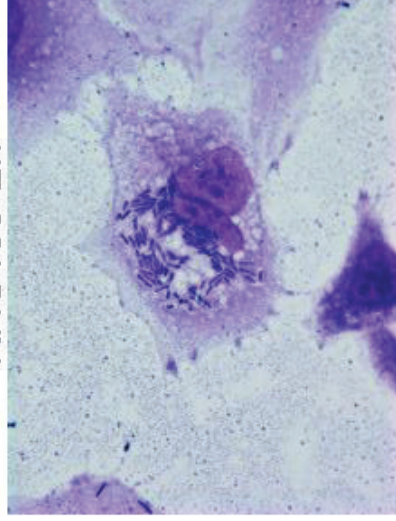
3. YciB may play an essential role in intracellular replication and/or intercellular spread

The *yciB* mutant KHS103, the wild-type parental strain O4576S1-G, and the mutant complemented with pND55 (*yciB*) in the presence of 1 μ M IPTG were compared in the invasion assay. Figure 9 shows two representative images per strain at 2, 4, 6, and 8 hours post infection. All three strains appear to grow at a similar rate during the first 2 hours after infection. At the 4 hour time point, the mutant has multiplied significantly less than either the wild-type strain or the mutant strain carrying the complementing plasmid. While both the wild-type strain and the mutant strain carrying the complementing plasmid show signs of intercellular spread at 6 hours post-infection, the *yciB* mutant strain does not. The number of bacteria in cells at the 8 hour time point varies too much for all three strains to draw any conclusions. This may partially be due to increased bacterial death in the overcrowded epithelial cells. Overall, the mutant appears to grow significantly slower than wild-type and shows no sign of intercellular spread.

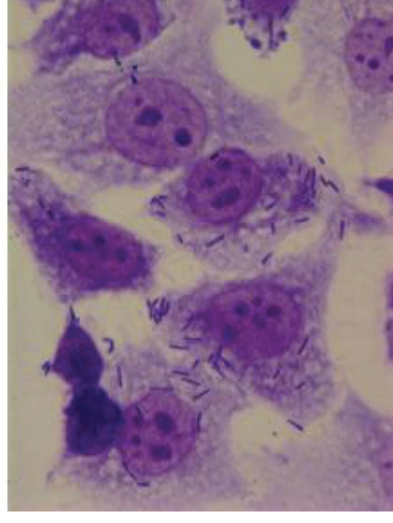
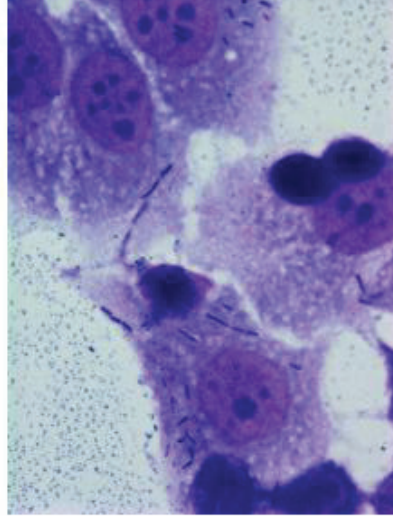
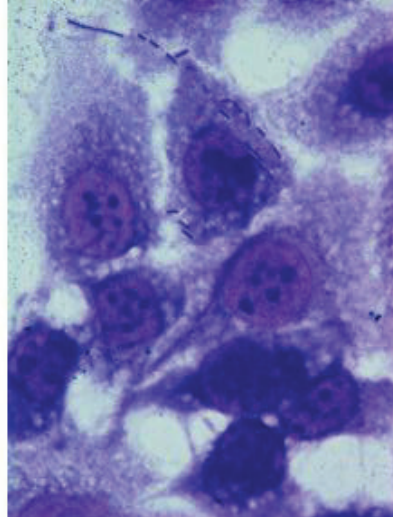
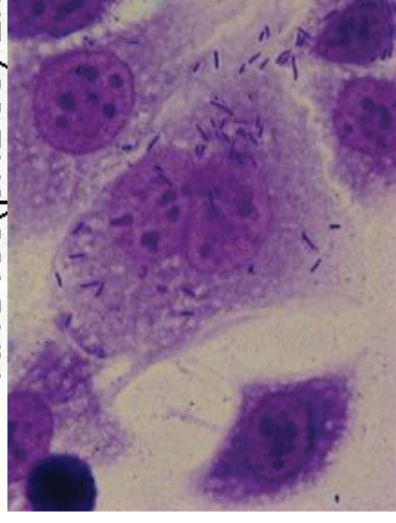
O4576S1G-2h



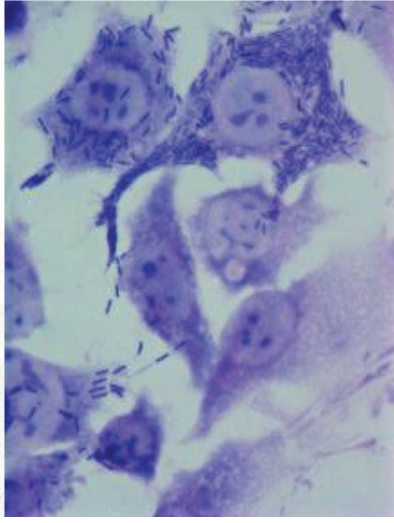
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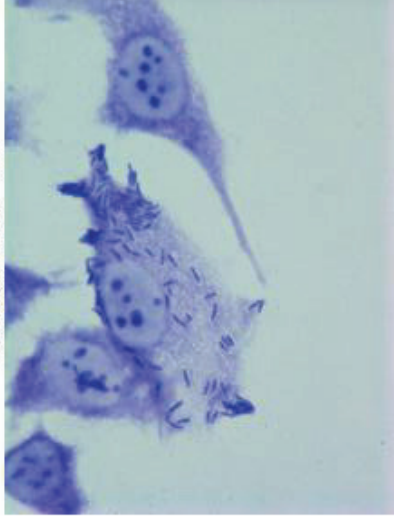
KHS103(pND55)-2h



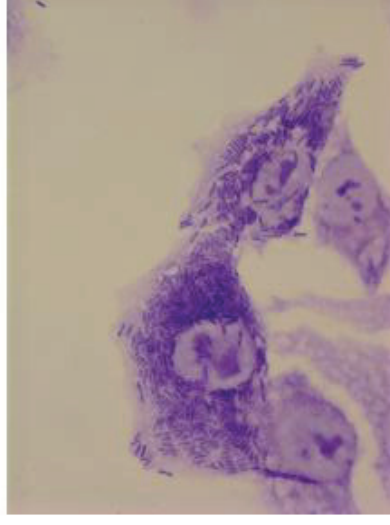
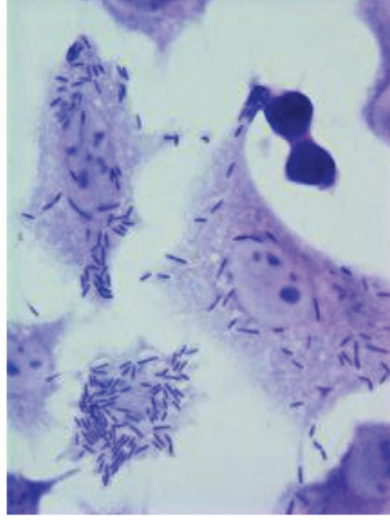
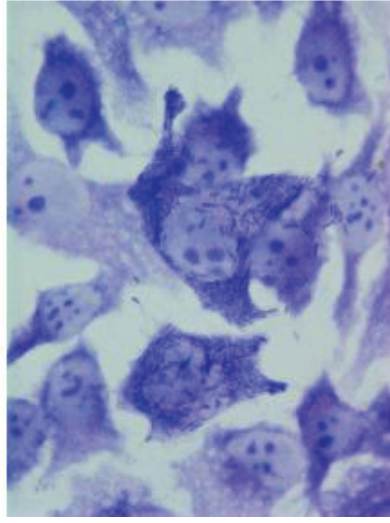
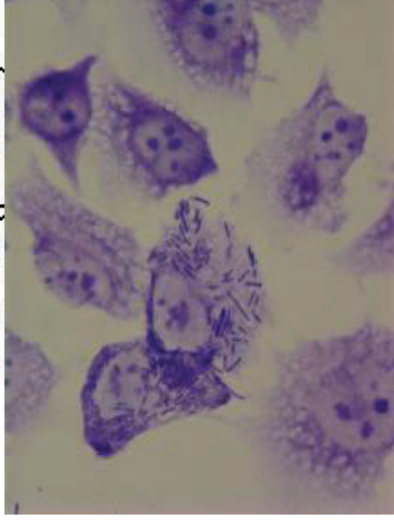
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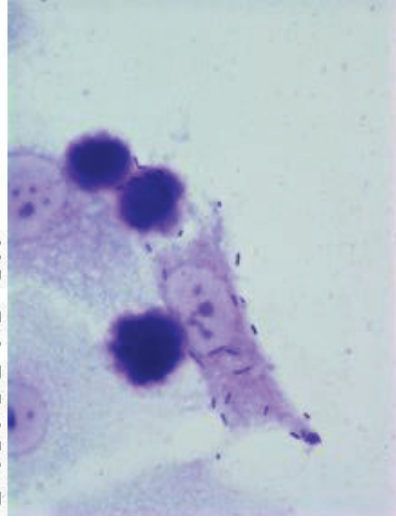
KHS103-4h



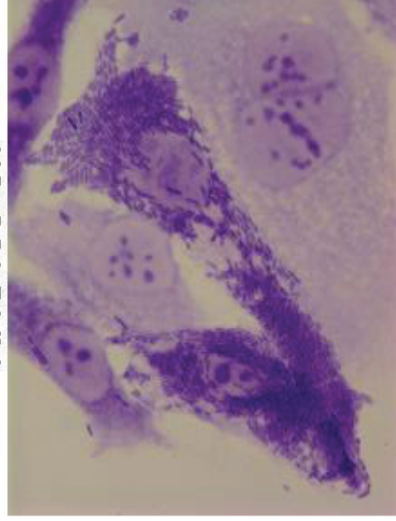
KHS103(pND55)-4h



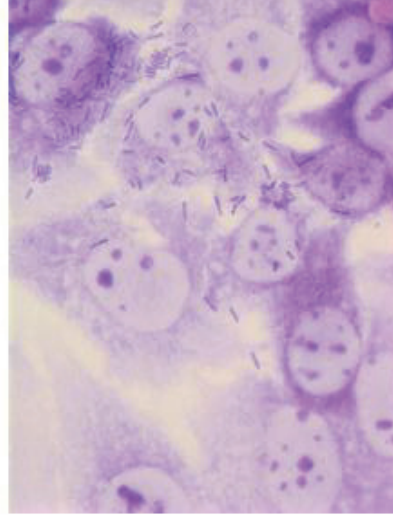
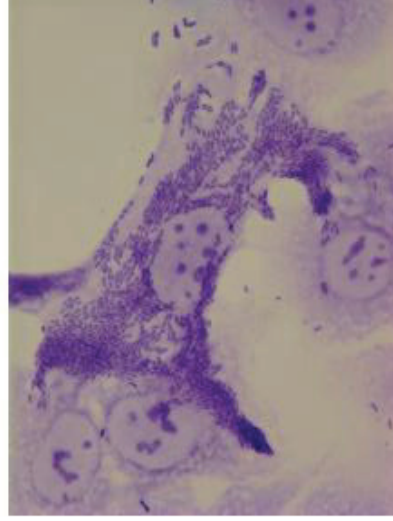
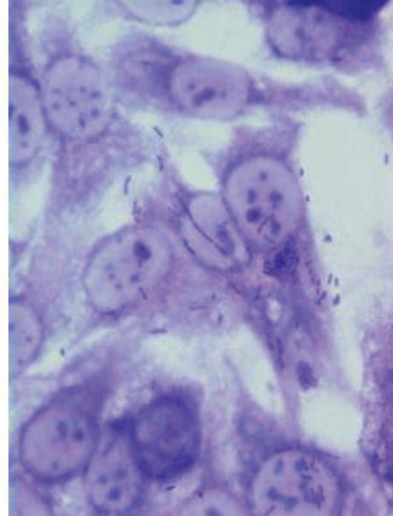
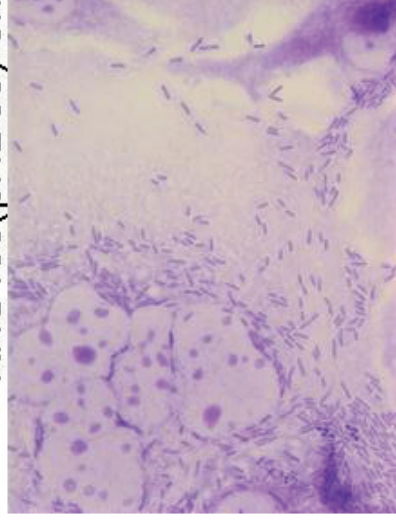
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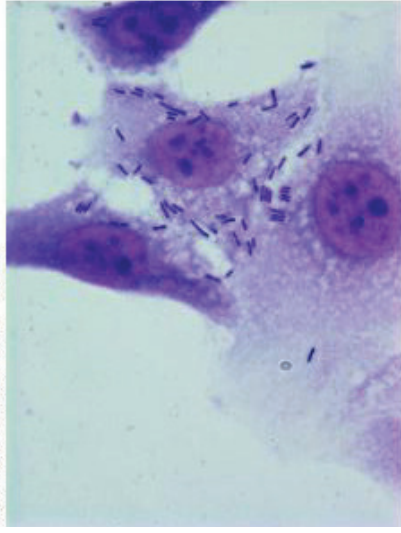
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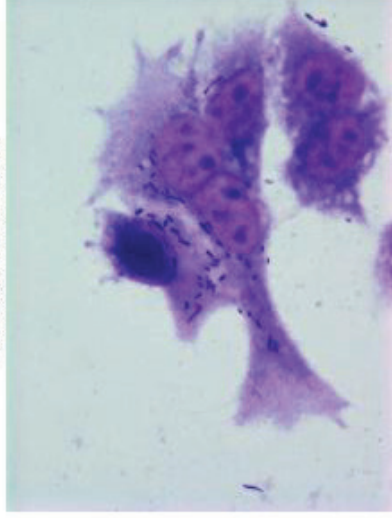
KHS103(pND55)-6h



O4576S1G-8h



KHS103-8h



KHS103(pND55)-8h

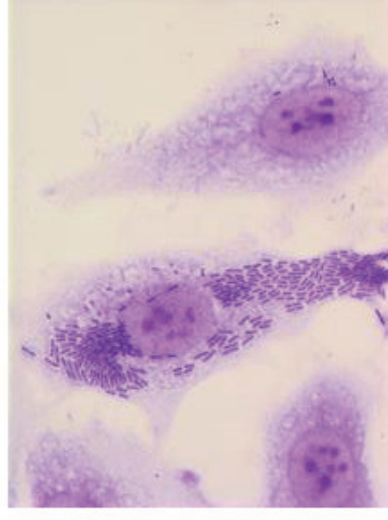
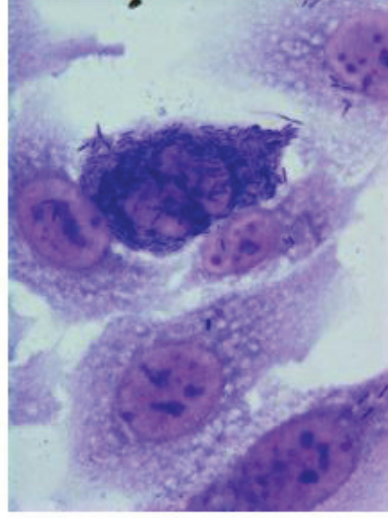
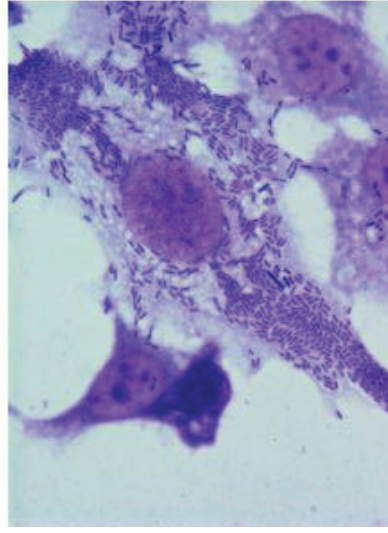
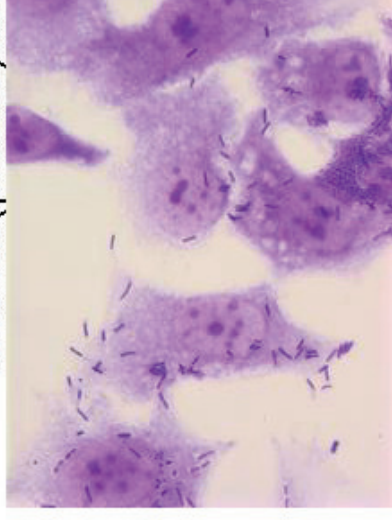


Figure 9: The *S. dysenteriae* *yciB* gene is essential for *in vivo* intracellular replication and spread

No growth defect is apparent in the *yciB* mutant 2 hours post-infection. At the 4 hour time point the mutant has replicated significantly less than the wild-type strain and the complemented mutant. Both the wild-type strain and the mutant carrying a complementing plasmid show signs of intercellular spread at the 6 hour time point.

4. *A S. dysenteriae yciB* mutant shows no *in vitro* growth defect

a. **Comparison of growth rates of *S. dysenteriae yciB* mutant and parental strain in LB broth showed no significant difference**

In vitro growth of KHS103, O4576S1-G, and KHS103 complemented with pND55 with varying amounts of IPTG were compared in an end point analysis and in a growth analysis. The *yciB* mutant did not show an *in vitro* growth defect compared to the parental strain O4576S1-G, and expression of pND55 with varying amounts of IPTG did not cause a significant difference in growth (Fig. 10, 11). This suggests that *yciB* expression is only important for *in vivo S. dysenteriae* growth.

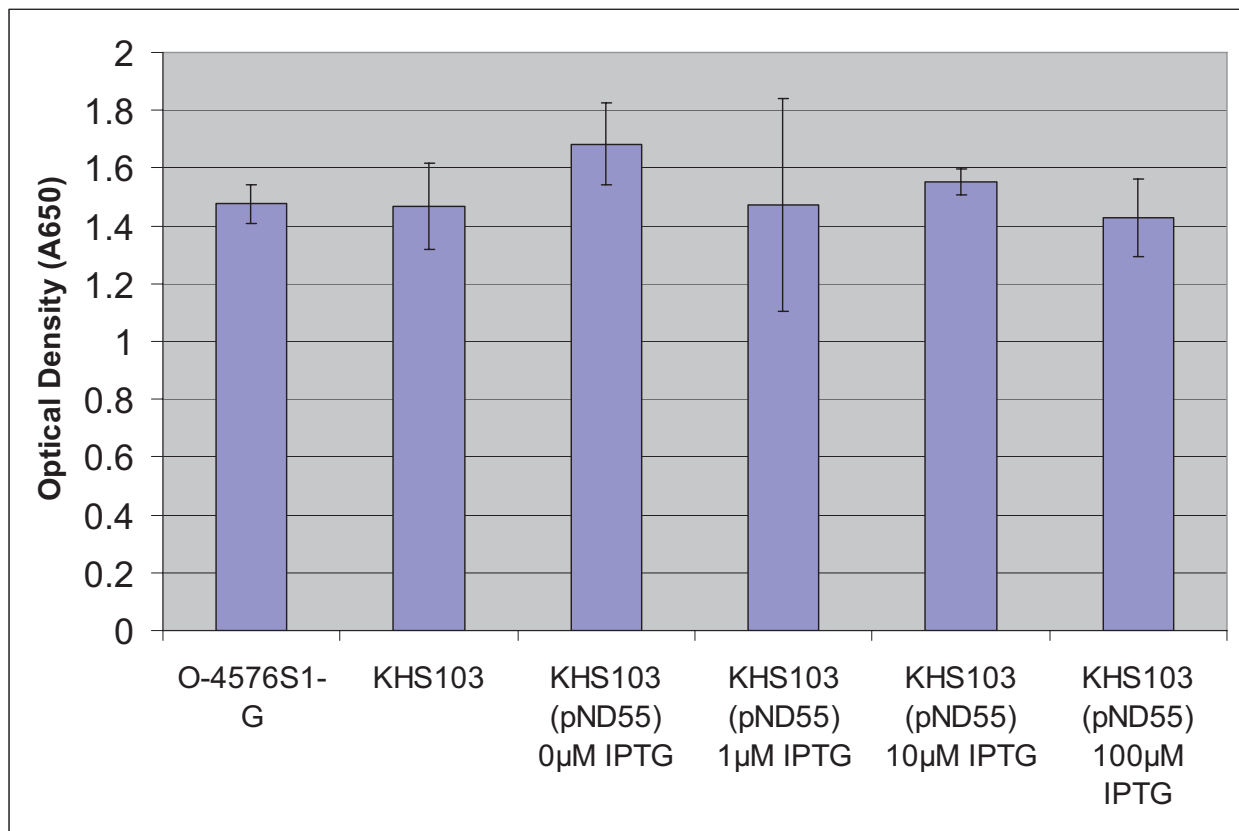


Figure 10: The *S. dysenteriae* protein YciB does not affect *in vitro* growth

No growth defect of KHS103 versus O4576S1-G was observed in an end point analysis. No significant difference in growth between KHS103, O4576S1-G, and KHS103 complemented with pND55 in the presence of 0, 1, 10, or 100μM

IPTG was observed. This suggests that YciB is not essential for *in vitro* growth of *S. dysenteriae*.

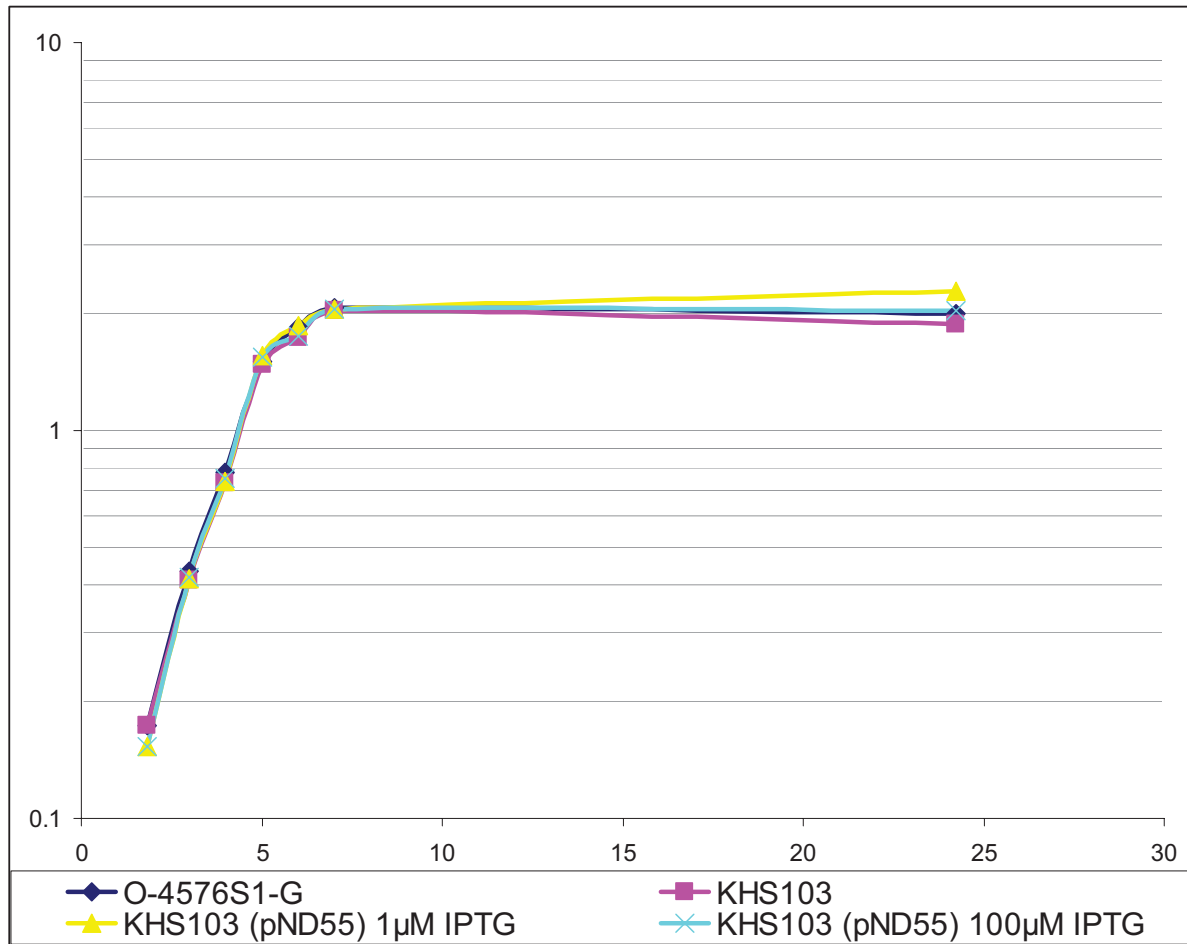


Figure 11: YciB does not affect *in vitro* growth rate

KHS103, O4576S1-G, and KHS103 complemented with pND55 in the presence of 1μM IPTG and 100μM IPTG showed no significant difference in growth rates. This suggests that YciB doesn't play an important role in *in vitro* growth.

b. Comparison of growth of *S. dysenteriae yciB* mutant and parental strain on LB agar showed no significant difference

Comparing the average colony size of KHS103, O4576S1-G, and KHS103 complemented with pND55 further suggested that YciB has no *in vitro* growth effect. The complemented strain was grown on various amounts of IPTG to test if altered expression of *yciB* resulted in a growth defect. Both KHS103 and O4576S1-G were grown in the presence of no IPTG and 5 μ M IPTG as controls. While supplementing the complemented KHS103 strain with 100 μ M IPTG resulted in significantly smaller colonies compared to no IPTG supplement, all strains, including the controls, formed smaller colonies on 5 μ M IPTG, suggesting that the difference observed on 100 μ M IPTG was a function of increased IPTG amounts, not of increased expression of *yciB* (Fig 12). Thus, while the level of YciB is important for *in vivo* pathogenesis and therefore presumably growth, the level of YciB has no *in vitro* growth effect.

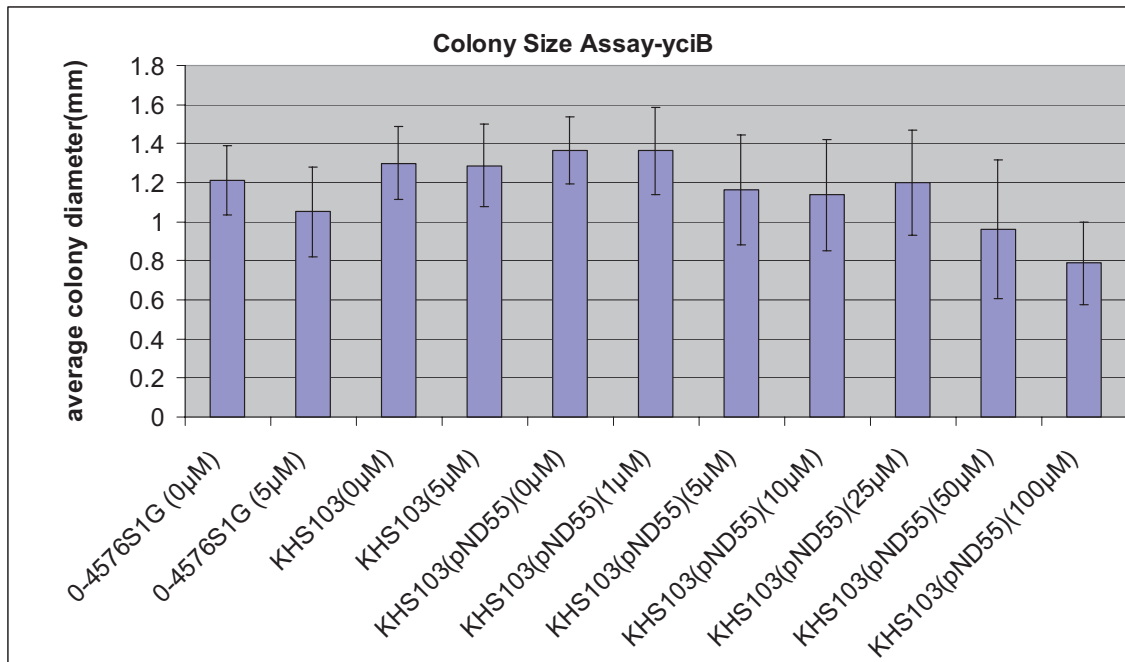


Figure 12: Varying the amount of YciB has no effect on colony size

The level of YciB has no effect on average colony sizes of KHS103, O4576S1-G, and KHS103 complemented with pND55.

IV. DISCUSSION

A. Identification of virulence gene *yciB*

The purpose of this project was to identify a novel virulence gene by analyzing the spontaneous, avirulent mutant SDU380. SDU380 had sustained a 33 kilobase deletion, and none of the genes in the deleted region were known *S. dysenteriae* virulence genes.

Three mutants (an oligopeptide permease mutant, a tryptophan biosynthesis mutant, and an *yciCBA* operon mutant) were constructed and tested for virulence in a plaque assay. The oligopeptide permease mutant KHS100 was able to form wild-type plaques, both in terms of number and in terms of size, indicating that the oligopeptide permease operon plays no role in *S. dysenteriae* invasion, intracellular replication, and intercellular spread, three essential steps in *Shigella* pathogenesis. Similarly, the tryptophan biosynthesis mutant KHS101 was able to form plaques. Though the wild-type parental strain formed slightly more and slightly bigger plaques, there was no significant difference in plaque formation, suggesting that the tryptophan biosynthesis operon plays no essential role in the three steps of *Shigella* pathogenesis. The *yciB* mutant KHS103 was, however, unable to form plaques, indicating that it is a virulence gene. Complementation of KHS103 and SDU380 with *yciB* restored the wild-type plaque phenotype, verifying that *yciB* is a virulence gene and indicating that *yciB* is sufficient to restoring plaque formation of SDU380. This further suggests that *yciB* is the only gene in the deleted region that plays a role in *S. dysenteriae* invasion, intracellular replication, and intercellular spread.

The expression level of *yciB* appears to play a role in *S. dysenteriae* virulence, as was shown by the loss of ability to form plaques of the complemented mutant when supplemented with high levels of IPTG. This was both true for complemented KHS103 and complemented SDU380, indicating that *yciB* expression is tightly controlled and consequently suggesting that

YciB is very important in *S. dysenteriae* pathogenesis. However, the levels of IPTG at which plaque formation of the mutant carrying a complementing plasmid could be restored were different in KHS103, which could form plaques when *yciB* was expressed with 0 μ M, 1 μ M, and 10 μ M IPTG, and SDU380, which could form plaques when *yciB* was expressed with 0 μ M and 1 μ M IPTG. This difference is likely due to genetic differences other than *yciB* between the two mutant strains. In other words, genes other than *yciB* in the deleted region of SDU380 affect virulence, but only *yciB* is essential in restoring ability to form plaques of SDU380.

B. Characterization of *yciB*

While the plaque assay is sufficient to identify a virulence genes, further studies are necessary to understand the nature of its involvement in virulence. An invasion assay was done to identify the stage of pathogenesis affected by loss of YciB. The data suggested that YciB affects intracellular growth and intercellular spread. This conclusion is based on the observation that at four hours post-infection infected eukaryotic cells contained fewer numbers of mutant bacteria relative to wild-type and mutant carrying a complementing plasmid. Furthermore, while both wild-type and mutant carrying a complementing plasmid showed signs of intercellular spread at the six hour time point, KHS103 showed no indication of spread. There is no evidence indicating whether loss of YciB affects these two steps of pathogenesis directly or indirectly.

While both *S. flexneri* and *S. dysenteriae yciB* mutants were defective in intracellular growth and intercellular spread, the two mutant strains displayed a different phenotype. The *S. flexneri yciB* mutant had a septation defect resulting in long filaments formation (12). No such defect was displayed by the *S. dysenteriae yciB* mutant. This difference is not surprising, since the two species, despite a similar general lifestyle and similar genetic makeup, are distinct.

The growth defect of the *S. dysenteriae yciB* mutant was further studied *in vitro*. Neither an endpoint analysis nor a growth curve verified the growth defect of KHS103 *in vitro*. While the colony size assay showed some indication that YciB affects replication rate, as seen by the decreasing average colony size with increasing amounts of IPTG and thus presumably *yciB* expression, the data were insufficient to conclude that the growth defect was solely due to YciB levels. A growth defect *in vivo* but not *in vitro* is not uncommon and suggests that YciB is regulated by a multitude of signals, including specifically intracellular signals.

While further analysis is necessary to identify the signals involved in YciB regulation and the extent of YciB involvement in *S. dysenteriae* virulence, this study identified a novel virulence gene in *S. dysenteriae*. The findings further suggest that YciB affects intracellular growth and intercellular spread, both essential steps in *Shigella* pathogenesis.

V. REFERENCES

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